RESEARCH, DISCOVERY & INNOVATION

Water & Energy Sustainable Technology Center

PROTOCOL

Protocol for the Evaluation of Virucidal Activity of Antimicrobial Coated Surfaces

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I. Overview

This document describes a testing protocol to support the registration of antimicrobial surface coating products with non-food contact disinfectant claims. The protocol is a hybrid of US EPA's Initial Virucidal Effectiveness Test using Feline Calicivirus as Surrogate for Norovirus[1] and EPA's 2016 Draft Protocol for the Evaluation of Bactericidal Activity of Hard, Non-porous Copper Containing Surface Products[2]. The following items summarize the approach employed to support these product claims:

1. This protocol is designed to address disinfectant claims for non-food contact coated surface products
2. The protocol is applicable to surface paint/coating products that are intended for indoor use only
3. Efficacy testing involves the evaluation of a production formulation against Feline Calicivirus
4. An effective product is expected to achieve a 3 $\log_{10}$ reduction (LR) in infectivity between treated test carriers and untreated control carriers within a 2 hr contact period
5. The protocol has been established for use with surface coating products, however, upon consultation with the EPA, the protocol may also be appropriate for testing other solid, non-food contact surfaces for antimicrobial activity
6. One representative paint sheen for wall and trim will be tested
7. The term "treated carriers" refers to coated carriers that contain the active ingredient (Corning® Guardiant® Antimicrobial Particles), while the term "untreated carriers" refers to coated carriers that do not contain the active ingredient.
8. Additional optional viruses (Appendix A) will be tested with one lot of treated test carriers and untreated control carriers prepared from base paint. Performance criterion will be a $\geq$ 99.9% reduction ($\geq$ 3 log reduction) in the numbers of each test virus. For microbes that recover only 3 logs on control coupons, performance criterion will be a $\geq$ 99% reduction with complete reduction of virus down to limits of detection.

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Table 1 provides an overview of carrier testing requirements.

Table 1. Carrier distribution of coated surface products

Product Lot	Paint	Test Carriers per Microbe	Control Carriers per Test Microbe
Lot I	Base paint	5 treated test carriers	3 untreated control carriers

II. Test Methodology

A. Materials

1. Coated test and control coupons
2. Challenge virus: Feline calicivirus Strain F-9; ATCC VR-782
3. Host cell: Crandel Reese Feline Kidney (CRFK) cells; ATCC CCL-94
4. Minimum Essential Medium (MEM) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS), 10µg/mL gentamicin, 100 units/mL penicillin and 2.5 µg/mL amphotericin B
5. Earle's Balanced Salt Solution (EBSS)
6. Fetal Bovine Serum (FBS)
7. Phosphate Buffered Saline (PBS)
8. Sephadex™/Sephacryl™ columns
9. Lab equipment and supplies

B. Carriers

1. From each production lot of base paint, prepare 5 treated test carriers and 3 untreated control carriers; extra carriers should be prepared for sterility assessment. The composition of the treated test carriers must be representative of the final product and meet the specifications for the target chemistry formulation. The chemical composition of the treated test product carriers must be documented.
2. Each lot of treated test and untreated control carriers is prepared by applying 2 coats of test or control paint on scrub test panels (such as BYK-Gardner 5015 Byko-chart, P121-10N). Each coat is applied at a thickness that is representative of anticipated final product using standard drawdown technique and allowed to dry for ≥24 hours. Additional coats/thickness can be applied based on use-conditions of the paint. These will be fully described and documented in the submission

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3. Cut coated test and coated control carriers into 1" x 1" square coupons from the center of the painted scrub test panels. Cut carriers are screened visually for surface defects and edge abnormalities. Discard carriers with visible surface or cut edge artifacts to ensure uniformity.
4. Each carrier will be physically screened to insure uniformity. Carriers with visible surface or edge abnormalities (chipping, gouges, pits or deep striations etc.) should be discarded. The screening will be conducted prior to the wear cycles
5. Uncoated scrub chart panels are used as an organism viability control
6. Place carriers in the biosafety hood for exposure to UV light for 5±2 minutes on each side to decontaminate the surface prior to testing. Place each carrier in a sterile Petri dish using forceps with the treated surface up
7. Provide details of physical screening and sterility check in the final report; coupon thickness and sheen will also be reported
8. Use decontaminated carriers within one week of preparation
9. All coupons are single use
10. Production lot (batch) identity will be maintained throughout the testing process

C. Preparation of test inoculum

Grow the FCV strain by inoculating confluent host cell monolayers (no more than 24-48 hours in age) using low multiplicity of infection (MOI).

1. Briefly, prepare a flask of host cells grown in cell culture media containing 10% fetal bovine serum (FBS). Wash cells three times with phosphate buffered saline (PBS) and inoculate with virus
2. Once the virus has adsorbed, wash the cell monolayer once in Earle's balanced salt solution (EBSS), re-feed with cell culture media and incubate
3. Cytopathic effects (CPE; small rounding of the cells with a slight granular look) starts to develop in 1-2 days following inoculation. Harvest when 80-90% percent of the monolayer shows cytopathic effects (CPE)
4. Transfer the contents into a centrifuge tube and centrifuge the suspension at 4 °C and 1000 x g for 15 min to separate the cell debris. The supernatant fluid is the stock virus for the test. Remove, aliquot and store in an ultra-low temperature freezer until the day of use
5. On the day of use an aliquot, thaw rapidly in a water bath at 37°C and refrigerate at 4°C until use in the assay.

Note: The percent FBS contained in the stock virus aliquot is adjusted to yield a 5% organic soil load.

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D. Efficacy Test Procedure

1. Evaluate 5 treated test carriers and 3 untreated control carriers
2. Control carriers should be evaluated concurrently with the test carriers
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a carrier
4. Record the initiation of the contact time and inoculate each carrier at staggered intervals with 20 µL of final test inoculum using a calibrated pipette (a positive displacement pipette is desirable)
5. Spread the inoculum across the surface of the carrier moving back and forth to ensure full coverage of the surface, spreading as close to the edge of carrier as possible using a bent pipette tip. Use an appropriate interval (e.g. 30 sec to allow enough time for careful spreading of the inoculum)
6. Place petri dishes with carriers in a controlled chamber set to 20°C and 50% RH with the lid on the Petri plate for 120 ± 5 min
7. Following the exposure period, pipette 1mL of MEM onto each test and control carrier. Using a sterile cell scraper, gently scrape the carrier. Continue pipetting the 1mL medium back-and-forth of the painted coupon to ensure the viral particles are homogenously mixed throughout the medium. Collect the medium-virus suspension and vortex to mix
8. Load each sample into individual pre-spun Sephadex™/Sephacryl™ columns. Viral suspension collected from surfaces will be passed through individual columns utilizing the syringe/plunger in order to remove cytotoxic leachates and neutralize active ingredient
9. Prepare serial 10-fold dilutions to be assayed for infectivity per Section E.

E. Infectivity assay

CRFK (feline kidney) cells; ATCC CCL-94 will be used as the indicator cell line for FCV infectivity assay.

1. Propagate cells in Minimum Essential Medium (MEM) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS), 10µg/mL gentamicin, 100 units/mL penicillin and 2.5 µg/mL amphotericin B
2. Seed cells into multi-well cell culture plates maintained at 36-38°C in a humidified atmosphere of 5-7% CO2. The cells are incubated for 3-5 days till confluency of cells is observed
3. Inoculate CRFK cultured cell monolayers with 200 µL (100-500 µL) the filtered suspensions. Each dilution is to be added to four wells containing confluent host-cell monolayers and incubated in a humidified atmosphere of 5-7% CO_2 at 36-38°C in for 5-7 days

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4. Score cultures periodically for 7 days for the absence/presence of cytopathic effects (CPE), cytotoxicity and viability. The CPE associated with FCV is visually evidenced under the microscope by the presence of small, shrinking cells that have detached from the monolayer.

Controls

1. Cell viability control

   Inoculate four wells with monolayers of host cells with cell culture media only and incubate in a humidified atmosphere of 5-7% CO_2 at 36-38°C in for 5-7 days. This control demonstrates that cells remain viable throughout the course of assay period.

2. Virus stock titer

   The Feline Calicivirus will be titered at the time of the test to determine the relative infectivity of the virus and to demonstrate the susceptibility of the host cells to support infection of FCV. Prepare serial tenfold dilutions in cell culture media. Inoculate select dilutions into four wells per dilution and incubate under the same conditions as the test.

3. Plate recovery control (PRC)

   Dry 20 µL of the virus on a 1" x 1" clean glass slide for 2 hours. Add 1mL of media and recover virus by same methodology used for coated test and control coupons. Pass suspensions through the Sephadex™/Sephacryl™ filtration columns and assay filtrate for infectivity of host cells. This control will determine the relative loss in virus infectivity resulting from drying and neutralization alone.

   The results from the plate recovery control will be compared with coated control results to compare recovery of virus on a non-porous surface vs. porous coated surface and confirm recovery of at least 4.8 $\log_{10}$ of infectious virus following drying and neutralization.

4. Cytotoxicity Control

   Inoculate one treated test carrier with 20 µL of Minimum Essential Medium (detailed previously) with 5% FBS soil load (no virus). Exposure conditions and time match those used for the test procedure. Following the exposure time, pipette a 1mL aliquot of medium and scrape the carrier surface with sterile plastic scraper. Gently rinse the surface repeatedly by pipetting the liquid volume and collect the medium. Vortex to mix and filter through Sephadex™ columns. Inoculate on host

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cell monolayers in a humidified atmosphere of 5-7% CO_2 at 36-38°C in for 5-7 days. Score for cytotoxicity.

5. Neutralizer effectiveness control (NEC)
   Prepare another set of cytotoxicity control dilutions as described. Prepare an additional set of indicator cell cultures and inoculate with a 100-500 µL aliquot of each dilution. Inoculate a 100 µL aliquot of low titer stock virus into each well and incubate as described for test and control plates. Dilutions that show virucidal activity will not be considered in determining reduction of the virus by the test substance.

Calculations

Calculate viral and cytotoxicity titers as $-\log_{10}$ of the 50 percent titration endpoint infectivity ($TCID_{50}$) as calculated by the method of Spearman Karber.

-Log of 1st dilution inoculated – [(Sum of % mortality at each dilution/100) – 0.5) x (logarithm of dilution)]

Log_{10} reduction achieved by the treated test carriers = (Average Log_{10} no. of organisms surviving on untreated control carriers) – (Average Log_{10} no. of organisms surviving on treated test carriers)

F. Product Performance Data

Mean log reduction data should be calculated and presented by comparing viable counts for the 5 treated test carriers and 3 untreated control carriers.

Acceptance Criteria: The acceptance criteria for the coated control carriers is 4.8 logs or greater of viable infectious units per carrier. All study controls must perform according to the criteria detailed in the study controls description section

Product Efficacy: For the test substance to be considered a disinfectant surface, a ≥ 99.9% reduction (≥ 3 log reduction) and/or complete reduction of infectivity should be measured (the difference between treated test carriers and the untreated control carriers).

G. Optional Label claims

The following label claims are supported by this protocol. Claims are limited to indoor use of hard, coated surface products.

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1. This surface kills at least 99.9% of viruses after a 2-hour contact time when maintained in accordance with the product care and use directions
2. Painted surfaces kill greater than 99.9% of [Feline Calicivirus] [COVID-19 virus] [Coronavirus 229E] [within] [after] [1] [2] hours of exposure
3. Kills [pathogenic] [disease causing] [harmful] viruses on [painted surfaces] *
4. Painted surfaces kill disease causing viruses [within] [after] 2 hours of exposure
5. EPA-registered paint that continuously kills 99.9% of Feline Calicivirus
6. Helps prevent the spread of viruses on painted surfaces
7. Kills at least 99.9% viruses* after a 2-hour contact time when maintained in accordance with the product care and use directions
8. [Antimicrobial][Microbicidal] paint
9. Painted surfaces continuously reduce viral contamination, achieving 99.9% reduction of viruses [within] [after] 2 hours of exposure, and continue to kill viruses even after repeated contamination
10. Microbicidal efficacy lasts as long as the integrity of the surface is maintained

**Feline Calicivirus*

Required Label Language

1. 1. Care and Use of Antimicrobial Copper Containing Surface Products in Health Care Facilities

"Product Care and Use: Antimicrobial copper containing surface products must be cleaned and disinfected according to standard practice. Health care facilities must maintain the product in accordance with infection control guidelines; users must continue to follow all current infection control practices, including those practices related to disinfection of architectural painted surfaces. This copper surface material has been shown to reduce microbial contamination but does not necessarily prevent cross contamination. This product must not be waxed, painted, lacquered, varnished, or otherwise coated by any other material."

1. Care and Use of Antimicrobial Copper Containing Surface Products for Non-Health Care Facilities

"Product Care and Use: Routine cleaning to remove dirt and filth is necessary for standard hygiene and to assure the effective antibacterial performance of the antimicrobial copper containing surface products. Gentle cleaning agents typically used for architectural painted surfaces are permissible. The use of an antimicrobial copper surface does not replace standard good hygienic practices and/or infection control procedures. This product must not be waxed, painted, lacquered, varnished, or otherwise coated by any other material."

Appendix A

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Methodology for optional additional viruses

Optional additional viruses will be tested using same methodology as described for Feline Calicicvirus. One lot of base paint will be used for evaluations. Modifications may be required such as a higher inoculum volume (as suggested in table 1) or a shorter exposure time than 2 hours to to ensure adequate infective virus survival on coated control coupons for the duration of test. Fetal Bovine Serum (FBS) load will be adjusted if inhibitory to viruses[3].

Table 1. Viral test strains, host cells and inoculum volumes

Virus	Cell Line[3]	Inoculum volume	Exposure Time
Adenovirus Type 4 (VR-4) or Type 5 (VR-5)	293 (CRL-1573), A-549 (CCL-185), HeLa (CCL-2), Vero (CCL-81)	20 µL	2 hours
Murine Norovirus	RAW 264.7 (TIB-71)	20 µL	2 hours
Influenza A (H1N1), ATCC VR-1469	Madin Darby Canine Kidney Cells (MDCK), ATCC CCL-34	20 µL; may require going up to 60 µL	1-2 hours
Influenza A (H3N2), Hong Kong Strain (VR-544)	Madin Darby Canine Kidney Cells (MDCK), ATCC CCL-34	20 µL; may require going up to 60 µL	1-2 hours
Rhinovirus 16, ATCC VR-283; Rhinovirus, type 37, strain 151-1 (VR-1607); Rhinovirus type 14 (VR-284)	H1 Hela ATCC CRL-1958, MRC-5 (CCL-171), WI-38 (CCL-75), H1-HeLa (CRL-1958)	20 µL	2 hours
Coronavirus 229E	WI-38 (human lung cells) ATCC CCL-75	60 µL	1-2 hours
SARS-CoV-2	Vero E6 cells, ATCC CRL-1586[4]	60 µL	1-2 hours
Human Coxsackievirus B3, Strain Nancy, ATCC VR-30	Vero, ATCC CCL-81	20 µL; may require going up to 60 µL	1-2 hours

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Rotavirus, Wa strain, (VR-2018)	Rhesus monkey kidney (MA-104) (CRL-2378) or African green monkey kidney - CV-1 (CCL-70)	20 µL; may require going up to 60 µL	1-2 hours
Hepatitis A virus, HM-175 strain (VR-2093)	Rhesus Monkey Kidney – FRhK-4 (CRL-1688)	20 µL; may require going up to 60 µL	1-2 hours
Herpes Simplex Virus, Type 1, strain F (1) (VR-733)	Vero (CCL-81), HEp-2 (CLL-23), primary rabbit kidney	20 µL; may require going up to 60 µL	1-2 hours
Respiratory Syncutial Virus, Long strain (VR-26)	HEp-2, MRC-5 (CCL-171), HeLa (CCL-2)	20 µL; may require going up to 60 µL	1-2 hours
Canine Parvovirus, Cornell-780916-80 strain, (VR-2017	A-72 (CRL-1542)	20 µL; may require going up to 60 µL	1-2 hours
Cytomegalovirus, strain AD-169 (VR-538)	MRC-5 (ATCC CCL-171) orWI-38 (CCL-75)	20 µL; may require going up to 60 µL	1-2 hours

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References

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